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PROTEIN COMPLEMENTATION

5. Name of your agent *(if you have one)*
KILBURN & STRODE

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Kilburn & Strode

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PROTEIN COMPLEMENTATION

This invention relates to pairs of parent plants for producing hybrid seeds and to methods for producing plants with a desired phenotype. The desired phenotype is an active enzyme, a regulatory protein or a protein which affects the structural integrity of a cell. Preferably, the desired phenotype is substantially absent from the parent plants/lines. In particular, the invention relates to parent plants and methods involving plant lines for producing male-sterile plants and seeds.

The present invention describes a protein complementation system, with a variety of different applications. The system can be explained and exemplified with reference to obtaining male-sterile plants and embryoless seeds although it is not limited to these applications.

The use of dominant Artificial Male Sterility (AMS) in plants is described in WO95/20668. This document describes a binary system using two genes which together (but not in isolation) cause male sterility. The genes are brought together by crossing plants, each parent being homozygous for the gene, which generates a homogenous population of male sterile plants. WO95/20668 describes several ways to implement the gene binary system, including the following:

i. a system based on activation of transcription: a transcriptionally inactive AMS gene is activated upon crossing by provision of the relevant transcription factor;

ii. a system based on activation of splicing: an AMS gene inactivated by the presence of an intron is activated upon crossing by provision of the relevant maturase;

iii. a system based on the suppression of a stop codon during translation: an AMS gene inactivated by introducing an artificial stop codon in the ORF, is activated upon crossing by provision of an artificial stop suppressor tRNA for the introduced stop codon.

iv. a system based on sequence-specific gene inactivation: One parent contains a modified male fertility gene and a transgene which inactivates only the unmodified male fertility gene. The other parent contains a transgene which inactivates only the modified male fertility gene. In the hybrid both the modified and unmodified male fertility genes are inactivated causing male sterility.

v. a system based on preventing restoration of male fertility by a restorer gene: the first parent contains the AMS gene and the restorer gene, and the second parent contains a gene inhibiting the action of the restorer gene.

However, the binary systems described above have so far proved complex to implement and have encountered a variety of difficulties.

For example, it has been found that the use of a suppressor tRNA (described in Betzner *et al.* 1996, Abstract of the 14th International Congress of Plant

Reproduction, Lorne, Australia) can have deleterious consequences for some plant species. While this does not preclude its use, it does make the screening of suitable transgenic plants more labour intensive than desirable. Another example is the leakiness of the T7 promoter (described in EP-A-0589841). Some plants transformed with a T7 promoter driving barnase were sterile in the absence of the T7 RNA polymerase. Again, this does not preclude use of the system but it does make it difficult to identify suitable transgenic plants. Furthermore, in certain plants the gene binary system is sub-optimal since not all of the required genetic elements are fully characterised.

Two areas of prior art have been explored which have resulted in a phenotype conferred to a plant by the combination of two proteins.

In 1989, Hiatt and coworkers (*Nature*, vol. **342**, p. 76-78) described the production of a functional antibody in tobacco by crossing tobacco plants expressing a gamma immunoglobulin gene and a kappa immunoglobulin gene.

Problems were, however, encountered with this system. Since the light and heavy chains of an antibody interact through disulfide bridges, the bridges were unable to form in the reducing environment present in the cytoplasm. Assembly of a functional antibody in plants thus requires that both chains are targeted to the endoplasmic reticulum then secreted to the apoplast (the space between cells). The production of antibodies in plants has thus been limited to the production of secreted antibodies or the production of single chain antibodies.

In 1992 Lloyd *et al.* (*Science*, vol. **258**, p. 1773-1775) described the transfer in Arabidopsis and tobacco of two maize genes coding for the transcription factors R and C1. Ectopic expression of these genes separately in heterologous plants has some effect on the transcription of endogenous genes. In particular the genes have some effect in isolation, and this may preclude their use for applied purposes. Co-expression of the two genes had more dramatic qualitative and quantitative effects, than expression of either gene alone. However, these genes have properties severely limiting their usefulness and their general inapplication is described in the paper.

It has been shown that the Arabidopsis transcription factors Apetala3 and Pistillata can be ectopically co-expressed, and jointly in concert cause a new phenotype in the Arabidopsis flower (Krizek and Meyerowitz, 1996, *Development*, vol. **122**, p. 11-22). The limitations described above for the R/C1 proteins also apply in this case.

The present invention describes a protein complementation system which overcomes many of the problems and difficulties associated with known gene binary systems. The protein complementation system according to this invention is based on the expression of two or more gene sequences in a single plant, which polypeptides/proteins, associate, interact or come together to form an active enzyme, a regulatory protein or a protein which affects the structural integrity of a cell. Some polypeptides/proteins may fall in more than one of these categories None of the individual gene sequences present in a given plant lead to a significant phenotypic effect in these plants.

The present invention describes the creation of a plant which has a desired phenotype through expression of an active enzyme, regulatory protein or protein which affects the structural integrity of a cell (eg. a membrane destabilizing protein). The plant may be obtained by crossing a pair of parent plants a and b. Plant a contains one or more gene sequences which encode a polypeptide(s) or protein(s) (A) with little or no activity so that the desired phenotype is not significantly (or substantially) caused by expression of the one or more genes in plant a alone. Plant b also contains one or more gene sequences which encode a polypeptide(s) or protein(s) (B) also, with little or no activity so that the desired phenotype is not significantly (or substantially) caused by expression of the one or more genes in plant b alone. When plants a and b are crossed, the resulting hybrid expresses both polypeptides and/or proteins A and B. These two polypeptides/proteins associate, interact or come together to form an active enzyme, regulatory protein or protein which affects the structural integrity of the cell, with the result that the daughter plant displays the desired phenotype. NB: From hereon, when discussing the polypeptides/proteins A or B they will be referred to only as 'polypeptides' for the sake of convenience.

This protein complementation binary system is simpler than the previously described binary systems since there is no need for interaction between genes, no required modification of the expression of genes and no modification of the level of expressed polypeptides in the daughter plant compared to the parent plants.

The present invention is described with reference to the Figures which are:

FIGURE 1A; Barnase coding sequence;
FIGURE 1B; Intergenic sequence;
FIGURE 1C; Barstar coding sequence;
FIGURE 2; Schematic illustration of pepA* and pepB* construction by Inverse PCR (IPCR)
FIGURE 3A; *In vitro* construction from synthetic oligonucleotides of S-peptide, S(+5)-protein and S-protein;
FIGURE 3B; *In vitro* construction from synthetic oligonucleotides of the sequence encoding the S-peptide and the (Gly4-Ser)3 linker;
FIGURE 4A; protein and DNA sequences of S-peptide and S-peptide with (Gly4-Ser)3 linker;
FIGURE 4B; protein and DNA sequences of S(+5)-protein and S-protein.
FIGURE 5; production scheme for embryoless maize grains.

According to a first aspect of the invention there is provided a pair of parent plants for producing seeds comprising:

(i) a first parent plant containing one or more gene sequences encoding a polypeptide A; and
(ii) a second parent plant containing one or more gene sequences encoding a polypeptide B;

wherein the polypeptides A, B, when expressed separately in different plants, do not form an active enzyme a regulatory protein or other protein which affects the structural integrity of the cell but when expressed in the same plant do form an active enzyme, regulatory

protein or other protein which affects the structural integrity of the cell. Presence of the active enzyme, regulatory protein or protein which affects the structural integrity of the cell in a single plant, is the desired phenotype.

The present invention includes the scenario of inter-extra-genic repression/complementation/suppression; that is, where a mutation in one subunit of a multi-subunit complex can complement a mutation in another sub-unit in order to restore the active enzyme, regulatory protein or protein affecting the structural integrity of the cell. In such a scenario, the polypeptide(s)/protein(s) A and B may be the same in the two parent plants, with the exception of the different mutations. Examples include the *E.coli* regulatory proteins as described by Tokishita S.I., and Mizuno T., 1994, *Mol. Microbiol. (UK)*, **13/3**, 435-444 and the GroES and GroEL proteins of *E.coli* as described by Zeilstra-Ryalls J., *et al.*, 1994, *J. Bacteriol. (US)*, **176, (21)**, 6558-65.

In the present invention, the pair of parent plants can be described as a pair of complementary plants for producing hybrid seeds or even a pair of complementary transgenic plants for producing transgenic hybrid seeds.

It is most likely that at least one of the pair of parent plants is transgenic. When used herein the term 'transgenic' refers not only to genetic material from another species but to genetically manipulated DNA from the same plant or species. The genetic manipulation of the plant may be by a microbiological process such as *Agrobacterium tumefaciens* (Horsch R.B., Fry J.E., Hoffman N.L., Eichholtz D., Rogers S.G., Fraley R.T., (1985),

Science, **227** : 1229-1231)). Alternative manipulations include biolistic transformation, a technique also well known in the art, the use of *Agrobacterium rhizogenes*, particle gun, electroporation polyethylene glycol or silica fibers.

The present invention may be applied to any plant, in particular, maize, wheat, tomato, oilseed rape, barley, sunflower, linseed, peas, beans, melon, pepper, squash, cucumber and egg plant (aubergine) and other broad acre plants.

Use of the term "one or more gene sequences encoding a polypeptide...." refers to any number of stretches of genetic material (preferably DNA) which can encode one or more peptides/polypeptides/proteins. Thus "polypeptides" A or B can actually comprise more than one amino acid sequence which may or may not be linked or associated. There is no restriction on the location in the parent plant genome of the one or more gene sequences. Where more than one gene sequence is present, encoding for more than one peptide/polypeptide/protein, the relationship between the encoded sequences in each parent plant is only relevant to the extent that the parent plant does not display the desired phenotype (to any significant level). When the one or more gene sequences encoding a polypeptide A are expressed in the same plant as the one or more gene sequences encoding polypeptide B, then the result, according to the invention is the phenotype of an active enzyme, a regulatory protein or a protein which affects the structural integrity of a cell. Proteins which affect the structural integrity of a cell include proteins that destabilise or create holes or ion channels in cellular membranes.

A particular application of the present invention is the production of male-sterile plants. Accordingly, the polypeptides A, B when expressed in the same plant may cause male-sterility by ablation of the tapetum. An alternative application, also of the first aspect of the invention is the expression of polypeptides A, B in the same plant which form an active enzyme, a regulatory protein or protein which affects the structural integrity of a cell, which, through cell ablation in a specific tissue results in a different phenotype, as described below.

In addition to causing male-sterility, potent hydrolases like barnase can be used for other applications where cell ablation is needed, for example to remove an unneeded organ from a hybrid crop. This may contribute to reducing downstream processing costs. One example is the production of embryoless seeds, which is now described as follows: In the production of flour (from wheat) or semolinas (from maize or wheat) or corn flakes (from maize) or for other uses, it would be desirable to have seeds with no embryo. The use of embryo specific promoters in the first aspect of the invention above would enable ablation of embryos in seeds, in a cross dependent manner. That is, in the seeds produced by the plant containing one or more gene sequences encoding polypeptide A, pollinated with pollen from a plant containing one or more gene sequences encoding polypeptide B. Self pollination of plant a has to be prevented, for example by making plant a male-sterile. A possible production scheme for valuable embryoless maize grains would be the following: generate a plant containing one or more gene sequences encoding polypeptide A (plant a) and a plant containing one or

more gene sequences encoding polypeptide B (plant b), designed so that combination of polypeptide A and polypeptide B in one seed results in embryo ablation. Figure 5 shows a production scheme for embryoless maize grains according to the invention.

The biochemical composition of plants can also be manipulated according to the first aspect of the invention, for example by fatty acid biosynthetic enzymes. Where the presence of an unusual but valuable fatty acid in the plant has a deleterious effect on the plant, it would be useful to be able to produce seeds with the unusual (fatty acid) oil through a cross between two lines having a normal (or quasi normal) oil composition (to the extent that each parent line is not deleteriously effected). Splitting the enzyme responsible for the valuable fatty acid biosynthesis in two or more inactive parts, provides a practical way of producing the seeds with the valuable oil. Where the enzyme responsible for the desired trait is heteromultimeric, separating the genes from the various monomers in the two parent plants is a simple way to implement the invention. More generally, this invention can be used to obtain hybrid seeds or hybrid plants with a particular phenotype which neither parent has. In particular, this invention can be used to create hybrid plants, resistant to a herbicide, via the crossing of two parent plants. Each of the parent plants expresses one or more non-functional parts of an active enzyme, regulatory protein or protein which effects the structural integrity of a cell, which is directly or indirectly responsible for herbicide resistance. As the one or more genes in each parent plant responsible for the trait will segregate independently, this will result

in the gametes of such hybrid plants (especially pollen grains) giving rise to a lower transfer of the herbicide resistance trait to relatives or to weeds (in comparison with a classical single gene). If the hybrid seed is the harvested desirable product, expression of the desired trait would be restricted to the seed endosperm and embryo since these tissues are genetically hybrids.

The active enzyme, regulatory protein or protein which affects the structural integrity of a cell is preferably localised to a tissue specific (ie. present only in a selected tissue). This requires that one or both of the gene sequences encoding the polypeptides A, B are operatively linked to an appropriately stimulated promoter, eg. a tissue specific promoter so as to produce the desired phenotype. Where only one of the polypeptides is limited to expression in a selected tissue, the other polypeptide requires constitutive expression or at least an expression pattern which overlaps with that of the first polypeptide.

As described above, the expression may be seed or embryo specific and promoters for such tissue specificity are well known in the art. In the case of male-sterility, the promoter is preferably tapetum specific. Such promoters known in the art include the TA29 promoter (EP-A-0344029), the A9 promoter (Paul *et al* 1992, Plant Molecular Biology, vol. 19, p. 611-622) and the promoters described in WO95/29247. In order for heterozygous plants to have the desired phenotype, promoters must be active at the sporophytic level.

The choice of gene sequence for producing an active enzyme, regulatory protein or protein which affects the

structural integrity of a cell depends, of course, on the desired phenotype. Any gene sequence encoding an active enzyme, regulatory protein or protein which affects the structural integrity of a cell can be used provided that the protein activity can result from the association, interaction or combination of two or more polypeptides encoded by two or more gene sequences and that their activity can result in the desired phenotype. Immediately obvious proteins which can be suitable are those which are naturally encoded by two or more polypeptides and which self-assemble to form the final protein structure. The individual polypeptide units (subunits) should have no significant activity *in vivo*.

Suitable proteins for use according to the invention include natural heterodimeric proteins such as the C1-R maize proteins and the Apetala3-Pistillata (Ap3-Pi) *Arabidopsis thaliana* proteins. When present in the tapetum, the dimer protein Ap3-Pi can activate genes responsive to this transcription factor (which would normally be inactive because this transcription factor is normally absent from, or present at a low level in, the tapetum). The activated gene is preferably, but not necessarily, endogenous to the plant of interest. For example, expressing the dimer Ap3-Pi in the tapetum of maize will activate transcription of genes normally involved in flower development in other floral organs, and will prevent normal pollen maturation. The level of sterility of such a system can be improved by also engineering into the daughter plant a gene sequence which is affected by the produced active enzyme or regulatory protein.

One example is the introduction into one of the parent lines of a gene sequence from Barnase or PR-Glucanase under the control of the Apetala3 promoter (pApetala3). The Apetala3 promoter is responsive to the Ap3-Pi dimer and thus expression of the Barnase or PR-Glucanase protein occurs in the daughter plant. Such a system provides for the enhancement of plant male-sterility with the additional advantage of being under a strict control mechanism (via the pApetala3). Thus, the cause of the desired phenotype may be direct, ie. a direct result of the active enzyme, regulation protein or protein which affects the structural integrity of a cell, or may be indirect, ie. acting via an intermediate factor. Other transcription factors, for use in the invention, exist already as, or can be engineered to, a heterodimeric form, for example using the dimerisation domains described below. These include artificial transcription factors made by the association of a DNA binding domain and an activation domain of different origins.

An alternative use of the Apetala3-Pistillata system, is the complementation of mutations in sub-units of the proteins. For example, one parent plant may express both proteins but with a mutation in one or the other so that the plant does not have the active dimer. The other parent plant may also express both proteins, in this case, a mutation being in the other protein. The second parent plant would not express the active dimer. A cross between the two parent plants would result in expression of genes to produce an active dimer.

Ectopic expression of the subunits for these transcription factors can be used to modulate expression of their target gene and cause male sterility or other

traits (including pleiotropic effects) in a cross-dependent manner.

It is also possible to use, according to the first aspect of the invention proteins which have to be "artificially" split into two or more nucleic acid coding sequences. The resulting polypeptides/proteins must associate, assemble, interact or come together when expressed in the same plant to form an active enzyme, regulatory protein or protein which affects the structural integrity of a cell. Such artificial splitting of enzymes and proteins is today easily achieved by predicting where the protein can be split into two or more domains, for example predicting by structural biochemistry such as X-ray crystallography, functional protein analysis in mutants, structure prediction from sequence analysis or by limited proteolysis, amongst other techniques. In this way, the random coil or other suitable regions are identified as places where the protein may be split.

Examples of artificially split proteins include:

Barnase: This protein has been widely used to cause cell ablation, when expressed in specific tissues. Under the control of a tapetum specific promoter, expression of a Barnase gene causes male-sterility in many plant species (EP-A-0344029). It is known that the Barnase protein can be split into two polypeptides, which *per se* have no catalytic activity [*in vitro*]. When put together the two polypeptides can self-assemble to produce an enzyme whose product has RNase activity. (Sancho and Fersht, 1992, J.Mol.Biol., **224**, 741-747).

RNase A can also be used. It was shown, as long ago as 1959 (Richards and Vithayathil, J.Biol.Chem., **234**, 1459-1465) that RNase A can similarly be split by mild proteolytic treatment into two polypeptides which can then reassociate and produce an active enzyme.

In order to implement a system, according to the present invention, involving artificially split proteins, it may be necessary to design genetic constructs in order to express the polypeptides therefrom. In order to design the genetic constructs whose products will associate to form the active enzyme some modifications may be required, eg. adding a methionine codon in front of the ORF encoding the second half of the active enzyme and a stop codon after the ORF encoding the first half of the active enzyme. Such genetic construct design is commonplace and well known to the person skilled in the art.

The invention may also be practised by expressing two portions of two different enzymes that together give a different activity than either of the intact parent proteins.

Preferably, both parent plants are homozygous with respect to the gene sequences encoding polypeptide A or polypeptide B. Such genotypes ensure that all offspring will express the active enzyme, regulatory protein or protein which affects the structural integrity of the cell.

If one or more of the polypeptides (A or B) is/are small and there are doubts that any of them will be stable in a cell, it is possible to use a well-known system wherein

the small polypeptide is fused to a "carrier protein" which protects it from being degraded or increases its proteolytic stability, but retains its freedom to interact with the other polypeptide(s) to form the active enzyme, regulatory protein or protein which affects the structural integrity of a cell.

The carrier protein is chosen so that the polypeptides A or B are not affected by the fusion and may have an easily detectable activity *in vivo*. One suitable carrier protein is the β-Glucuronidase (GUS) protein, which tolerates addition to its NH_2 end, and is a good reporter gene in plants. In this case, one can use the level of GUS activity to evaluate the expression level of the fused small polypeptide. This can be useful for screening primary transformants.

In some cases, when expressed in two or more portions, the polypeptides may not spontaneously associate, assemble, interact or come together *in vivo* to reform an active protein, or regulatory enzyme or protein which affects the structural integrity of a cell. In other cases the association of the polypeptides may be weak so that little active reconstituted protein is formed. To circumvent these problems, each protein portion may be linked to a protein dimerisation domain, thus enabling the portions to be brought together *in vivo*. Such protein dimerisation domains are found in many proteins that naturally form dimers or multimers and the linking technique is well known in the art.

For example, the human cysteine-rich protein LIM double zinc finger motif has been fused to the Ga14 and VP16 proteins. In contrast to the unmodified Ga14 and Vp16

proteins the LIM-Ga14 and LIM-VP16 associate *in vitro* and *in vivo* (in NIH 3T3 mammalian cells) forming an active transcription factor (Feuerstein *et al.*, 1994, Proc.Natl.Acad.Sci. U.S.A. **91**, 10655-10659). The LIM motif is found in many organisms. For example, a sunflower pollen specific protein with a LIM domain has been identified (Batlz *et al.*, 1996, Plant Physiology (Supplement **III**, 59). Other protein dimerisation domains exist such as the leucine zipper (Turner, R. and Tijian R., 1989 Science, **243**, 1689-1694), the helix-loop-helix (Murre *et al.*, 1989, Cell, **56**, 777-783), the ankyrin Blank *et al.*, 1993, Trends in Biochemical Sciences, **17**, 135-140) and the PAS (Huang *et al.*, 1993, Nature, **364**, 259-262) domains.

One may also wish to ensure that the genes encoding polypeptides A or B are inserted in the genomes of parents a and b at an identical position (or at tightly linked positions) so that their chance of co-segregation in the transgenic hybrid is low. This can be advantageous, for example in the production of hybrid seed since the two genes that are used to create the male-sterile parent plant will subsequently segregate. Thus, F1 hybrid progeny are 100% male fertile since no hybrid plant can inherit both components of the male-sterility system.

The gene sequences carried by the parent plants a and b which encode part of the active enzyme, regulatory protein or protein which affects the structural integrity of a cell may be from a different organism. The gene sequences do not have to be plant derived and include genes from microbial or other sources. For example, the gene sequences may be Arabidopsis endogenous sequences in

maize or tomato parent plants. Also, the gene sequences may be those which, in combination with a tissue specific promoter, are expressed in a tissue in which the gene sequences are not normally expressed.

According to a second aspect of the invention there is provided a method for producing a plant having a desired phenotype of an active enzyme, a regulatory protein or a protein which affects the structural integrity of a cell, the method comprising crossing a first plant line with a second plant line wherein the first line contains one or more gene sequences encoding a polypeptide A which is part of an active enzyme, regulatory protein or protein which affects the structural integrity of a cell but which line does not have the phenotype and wherein the second line contains one or more gene sequences encoding a polypeptide B which is complementary to the polypeptide or protein A but which line does not have the desired phenotype. Here, the term "complementary" means that when expressed in the same plant the polypeptides A and B associate, interact or come together to form the phenotype of an active enzyme, a regulatory protein or protein which affects the structural integrity of a cell.

Such a method may incorporate one or more of the features described above for the first aspect of the invention and the invention contemplates the application of these aspects according to the second aspect of the invention.

According to a third aspect of the invention there is provided a seed or plant obtainable from a pair of plants according to the first aspect of the invention or by a method according to the second aspect of the invention.

According to a fourth aspect of the invention there is provided a seed or plant having a phenotype of an active enzyme, regulatory protein or protein which affects the structural integrity of the cell, which is caused by the combined action of two or more transgenes, the transgenes not being present on the same copy of a chromosome. The preferred embodiments of the first, second and third aspects of the invention also apply to the fourth aspect. This means that the two or more transgenes are either on different chromosomes, or on different copies of the same chromosome, ie. the plant is made by a cross.

The invention will now be described by the following non-limiting Examples:

EXAMPLE 1

Splitting the Barnase Gene into Two Components (Figure 1)

The results of Sancho and Ferscht, 1992, J.Mol.Biol., **224**, 741-747 show that Barnase activity can be obtained by combining a peptide A containing amino acids 1 to 36 of the mature Barnase protein and peptide B containing amino acids 37 to 110 of the mature Barnase protein. The allele of Barnase which is described in Sancho and Ferscht is a mutant which has a methionine at position 36, allowing cyanogen bromide to cleave between 36 and 37 and produce the 2 peptides. The following genetic constructs, to express the peptides, were prepared:

Peptide A:

i. A Barnase gene with a methionine codon (amino acid position -1) added before codon 1 of the mature Barnase sequence so that translation can take place

described in Paul *et al*, 1992, Plant Mol.Biol., **19**, 611-622.

ii. An ORF coding for a peptide called A*, containing a methionine followed by amino acids 1 to 35 of mature Barnase protein followed by an Ochre stop codon.

iii. A gene made of ORF A* under control of the A9 promoter by using IPCR on our plasmid p3079, which contains the AMS gene pA9-Barnase (as in i. above) - Barstar - CaMV 3' region. (See Figure 2).

Plasmid p3079 was constructed by cloning a fragment containing the ORFs for Barnase-Barstar, obtained by PCR from pWP127 (Paul *et al*, 1992, *supra*), in our plasmid p1415, which is a derivative of pWP91 (WO-A-9211379) where the EcoRV restriction site has been converted to HindIII. IPCR was then performed on p3079 using primers B3 and B4 (see Figures 1 and 2) designed so that the sequence between codon 36 of Barnase and stop codon of Barstar is not part of the amplified product. The IPCR amplified sequence was then circularised by ligation and the resulting plasmid was introduced into *E. coli* The plasmid was then prepared, cut with EcoRI and the fragment containing the ORF A* was cloned in the EcoRI sites of p1415, so that ORF A* would be under the control of the A9 promoter from a sequence not treated by PCR.

The resulting plasmid p2022 contains ORF A* in the A9 expression cassette.

Peptide B:

i. An ORF coding for a peptide called B* which starts with a methionine codon followed by codons 37 to 110 of the mature Barnase sequence. In effect this transfers the methionine 36 of the mutant Barnase gene (Sancho and Ferscht, 1992, *supra*) from peptide A to peptide B, yielding peptides A* and B*.

ii. Gene for ORF B* containing the ATG (amino acid position -1) of Barnase (in p3079) fused to codon 37 of Barnase, under control of the A9 promoter, by deleting (by IPCR with suitable primers) (see below)) codons 1 to 36 of the mature Barnase sequence.

This was done by performing on p3079 an IPCR reaction using primers B1 and B2, (Figures 1 and 2) designed so that the sequence between codon 2 and codon 36 of Barnase is not part of the amplified product (see Figure 2). The IPCR product is treated as described above for ORF A*, and cloned under control of the A9 promoter in p1415.

The resulting plasmid p2023 contains ORF B* - Barstar in the A9 expression cassette.

In Fig. 2: Circular plasmid p3079, containing the A9-driven barnase/barstar gene (Figure 1) in p1415, served as template for Inverse PCR. As the PCR primers (Figure 1) pointed into opposite directions, the IPCR yielded a linear double-stranded plasmid DNA from which the region in between the 5' ends of the annealed PCR primers was deleted (below). Intramolecular ligation would then

yield circular deletion plasmids which were introduced into *E.coli* for further subcloning.

Also In Fig. 2-:

lane 1:

A schematic (not to scale) representation is shown of plasmid p3079. The different structural parts of the coding regions are highlighted. ATG and TAA represent the start and stop codon of barnase and barstar, respectively. The relative positions of codons 35, 36 and 37 of the mature Barnase protein are indicated.

lane 2:

IPCR with primers B1 and B2 deleted codons 1 to 36 of the mature Barnase protein. Intramolecular ligation of the linear deletion plasmid then fused the ATG start codon to codon 37 yielding the pepB*/barstar region.

lane 3:

IPCR with primers B3 and B4 deleted the sequence downstream of the barnase codon 35 as indicated. Intramolecular ligation of the linear deletion plasmid then fused the barnase codon 35 to the barstar stop codon yielding the pepA* sequence.

EXAMPLE 2

Plant Transformation with the Genetic Constructs in Example 1

Genes pA9-A* and pA9-B* were cloned into derivatives of the plant transformation vector pBin19 Beven *et al.*, 1984, Nucl. Ac. Res. **12**, 8711 Frish *et al.*, 1995 Plant Mol. Biol., **27**, 405-409 and Arabidopsis plants containing pA9-A*, or pA9-B*, or both genes, were obtained. Plants

containing both genes were male sterile, whereas plants containing one gene were unaffected by the transgene. Plants with one gene were allowed to self, their progeny was harvested, and was analysed to identify homozygous and heterozygous T1 plants. T1 plants with pA9-A* were crossed with T1 plants with pA9-B*. The hybrid seeds obtained displayed the predicted phenotype: wild type if containing one gene only, and male sterile when containing the two genes.

Genes are introduced into maize and into tomato by biolistic or Agrobacterium-mediated transformation, and plants are regenerated and assessed for male fertility in a similar way. (Mornish *et al.*, 1990 Biol/Technology 8, 833-839 and Fillati *et al* ., 1987 Bio/Technology 5, 726-7390.

EXAMPLE 3

Splitting an RNAseA gene into two components (Figures 3 and 4)

From the work of Richards and Vithayathil (1959 *supra*), we know that the enzyme RNAseA can be cleaved (by the protease subtilisin) to generate two polypeptides: the S-peptide contains amino acids 1 to 20 of RNaseA, and the S-protein contains amino acids 21 to 124 of RNaseA. When combined, the S-peptide and the S-protein associate, and reconstitute an active enzyme. The last 5 amino acids of the S-peptide are not needed for reconstituting RNaseA: a smaller S-peptide made of amino acids 1 to 15 is sufficient. Genes which express the S-peptide and the S-protein under control of the A9 promoter were used to develop a system according to the invention.

The starting material was a synthetic gene coding for bovine pancreatic RNAseA (Vasantha and Filpula, 1989, Gene 76 53-60). A gene coding for the ORF of RNaseA was made using synthetic oligonucleotides (see Figures 3A and 3B). The nucleotide sequence of the gene was designed to be compatible with maize codon usage, according to Fennoy and Bailey-Serres, 1993 Nuc. Acids Res., 21, 5294-5300. We studied 2 possibilities for each polypeptide. PCR with suitable primers was used to amplify from the full length ORF the following sequences:

S-peptide:

i. A gene for the S-peptide containing a methionine translation initiation codon followed by codons 1 to 15 of the mature RNaseA sequence (see Figures 4A and 4B).

ii. A gene made of a methionine translation initiation codon followed by codons 1 to 15 of the mature RNAseA sequence, followed by a linker sequence encoding (Gly4-Ser)3 (see Figures 4A and 4B). This gene was designed so that it can be fused in frame to the ORF of the GUS protein by cloning in the BamHI site of plasmid p2027 which contains the GUS gene from pBI101.3 (Jefferson, 1987 Plant Mol.Biol.Reporter, 5 387-405).

S-protein:

i. A gene for the "S-protein +5", which contains a methionine translation initiation codon followed by codons 16 to 124 of mature RNAseA sequence.

ii. A gene for the S-protein which contains a methionine translation initiation codon followed by codons 21 to 124 of mature RNAseA sequence.

Genes under control of the A9 promoter were then built and introduced into plants as in Example 2.

In Fig. 3A: The sequences encoding the S-peptide, the S(+5)-protein and the S-protein were constructed by first aligning sense oligonucleotides RN-I to RN-VII (lanes 2, 5, 7, 9, 11, 13, 16) along complementary guide oligonucleotides RN-1 to RN-6 (lanes 3, 6, 8, 10, 12, 14) and then selectively ligating the correctly aligned sense oligonucleotides using Taq-DNA-Ligase.

The ligation resulted in a continuous single DNA strand (sense) which was subsequently amplified by Vent DNA polymerase (25 PCR cycles) using one of two primer pairs as follows: (i) Primers RN-a (lane 1) and RN-b (lane 15) amplified the full ligation product. The PCR product was gel purified and cleaved with restriction enzymes BamHI (underlined, lanes 1 and 15) and BgIII (underlined, lanes 2 and 4) to yield two DNA fragments encoding the S-peptide and the S(+5) protein. The two fragments were cloned separately into the BamHI site downstream of the pA9 promoter in plasmid p1415. (ii) Primers RN-d (lane 4) and RN-b (lane 15) amplified the coding sequence of the S-protein. The PCR product was cloned as described in (i).

lane 1: PCR primer (sense) RN-a
lane 2: Oligonucleotide RN-I and alignment to oligonucleotide RN-II
lane 3: Guide oligonucleotide RN-1 (antisense)

lane 4: PCR primer (sense) RN-d
lane 5: Oligonucleotide RN-II (continued from lane 2) and alignment to oligonucleotide RN-IIIN
lane 6: Guide oligonucleotide RN-2N (antisense)
lane 7: oligonucleotide RN-IIIN (continued from lane 5) and alignment to oligonucleotide RN-IV
lane 8: Guide oligonucleotide RN-3 (antisense)
lane 9: oligonucleotide RN-IV (continued from lane 7) and alignment to oligonucleotide RN-V
lane 10: Guide oligonucleotide Rn-4 (antisense)
lane 11: oligonucleotide RN-V (continued from lane 9) and alignment to oligonucleotide RN-VI
lane 12: Guide oligonucleotide RN-5 (antisense)
lane 13: oligonucleotide RN-VI (continued from lane 11 and alignment to oligonucleotide RN-VII
lane 14: Guide oligonucleotide Rn-6 (antisense)
lane 15: PCR primer (antisense) RN-b
lane 16: oligonucleotide RN-VII (continued from lane 13)

Symbols:
(5'): non-phosphorylated 5' end
(5P): phosphorylated 5' end
(30H): conventional 3' end
(small letters): bases added for the convenience of cloning.

In Fig. 3B: The sequences encoding the S-peptide with the $(Gly_4Ser)_3$-linker peptide were constructed by first aligning sense oligonucleotides RN-I and RN-VIII (lanes 2 and 4) along the complementory guide oligonucleotide RN-7, and then selectively ligating the correctly aligned oligonucleotides using Taq-DNA-Ligase.

The ligation resulted in a continuous single DNA strand which was subsequently amplified by Vent DNA polymerase (25 PCR cycles) using the primer pair RN-a (lane 1) and RN-c (lane 5). This PCR reaction yielded the full length, double stranded ligation product. The PCR product was gel purified, then cleaved with restriction enzymes BamHI (underlined, lane 1) and BglII (underlined, lane 5) and cloned into the BamHI site of p2027 to generate an NH_2-terminal protein fusion to GUS under the control of the pA9 promoter (p2027 was constructed by cloning the GUS coding sequence of pBI101.3 as a BamHI/SmaI fragment into the BamHI site of p1415).

lane 1: PCR primer (sense) RN-a
lane 2: Oligonucleotide RN-I encoding the S-peptide as in Figure 3a and the alignment to oligonucleotide RN-VIII encoding the (Gly4-Ser)3 linker peptide
lane 3: Guide oligonucleotide (antisense) RN-7
lane 4: Oligonucleotide RN-VIII (continued from lane 2)
lane 5: PCR primer (antisense) RN-c

Symbols:
(5'): non-phosphorylated 5' end
(5P): phosphorylated 5' end
(30H): conventional 3' end
(small letters): bases added for the convenience of cloning

EXAMPLE 4

Use of the Dimer Protein Apetala3-Pistillata

Apetala3 (Ape3) and Pistillata (Pi) are two proteins of *Arabidopsis thaliana* which are involved in the regulation

of floral differentiation. The genes are known while the endogenous pattern of expression in the tapetum is not known. Expression of the Arabidopsis genes in the maize tapetum leads to disruption of the normal anther development by activating normally silent genes. These genes can also be used to activate, in the maize tapetum, an Arabidopsis promoter responsive to the Ap3-Pi dimer such as the Ap3 promoter (pAp3) itself.

We have built the following genes:

pA9-Apetala3

The cDNA for Ap3 (Jack *et al*, 1992, Cell 68, 683-697 GenBank Accession No. M86357) was cloned in the A9 expression cassette of pWP91 (WO-A-9211379) giving plasmid p4796. This plasmid contains the Ap3 cDNA with approximately 15 bases of 5' untranslated sequence followed by the whole ORF (698 bases from ATG to TAA) followed by approximately 120 bases of 3' untranslated sequence, cloned in the BamHI site of pWP91.

pA9-Pistillata

The cDNA for Pi (Goto and Meyerowitz, 1994, Genes Dev. 8, 1548-1560 GenBank Accession No. D30807) was cloned in the A9 expression cassette of pWP91 (WO-A-9211379) giving plasmid p0180. This plasmid contains the Pi cDNA with approximately 24 bases of 5' untranslated sequence followed by the whole ORF (626 bases from ATG to TGA) followed by approximately 250 bases of 3'untranslated sequence, cloned in the XbaI-BamHI sites of pWP91.

pApetala3-PRGlucanase

The A9 promoter sequence in plasmid A9PR (described in Worrall *et al*, 1992, The Plant Cell, **4**, 759-771) was replaced by a 1250 bp (approx) sequence containing the Ap3 promoter region, obtained by PCR amplification of *Arabidopsis thaliana* genomic DNA, according to the published sequence (Jack *et al*, 1994 Cell, **76**, 703-716), giving plasmid p4817.

The genes were introduced in maize in various combination, by biolistic transformation techniques known in the art. Plants were regenerated and assessed for male fertility.

-p4796 (pA9-Ap3)/p0180 (pA9-Pi) cause male sterility. Neither of them alone causes male sterility.
-p4796/p0180/p4817 (pAp3-PRGlucanase) cause sterility, when p4817 with only one of the two transcription factor genes does not.

CLAIMS

1. A pair of parent plants for producing seeds comprising:

(i) a first parent plant containing one or more gene sequences encoding a polypeptide or protein A: and

(ii) a second parent plant containing one or more gene sequences encoding a polypeptide or protein B;

wherein the polypeptides A, B, when expressed in separate plants, do not form an active enzyme, a regulatory protein or protein which affects the structural integrity of a cell, but when expressed in the same plant do form an active enzyme, regulatory protein, or protein which affects the structural integrity of a cell.

2. A pair of plants as claimed in claim 1 wherein the one or more gene sequences from at least one of the parents is transgenic.

3. A pair of plants as claimed in claim 1 or claim 2 wherein the polypeptides or proteins A, B, when expressed in the same plant, cause cell ablation, especially male-sterility or embyroless seeds.

4. A pair of plants as claimed in any one of claims 1 to 3 wherein one of the parent plants is male-sterile.

5. A pair of plants as claimed in any one of claims 2 to 4 wherein the one or more gene sequences encoding both or one of the polypeptides or proteins A, B, is operatively linked to a tissue specific promoter.

6. A pair of plants as claimed in any one of claims 1 to 5 wherein the polypeptides A, B are naturally occurring subunits of the protein complex of an active enzyme, regulatory protein, or protein which affects the structural integrity of a cell.

7. A pair of plants as claimed in claim 6 wherein the polypeptides A, B are two polypeptide subunits of an enzyme having RNase activity such as the enzyme Barnase or RNase A or the monomers of the protein complex of the Apelata3-pistillata.

8. A pair of plants as claimed in any one of claims 1 to 5 wherein the polypeptides A, B are artificially split polypeptides of an active enzyme, regulatory protein or protein which affects the structural integrity of a cell.

9. A pair of plants as claimed in any one of the preceding claims wherein each parent plant is homozygous with respect to the one or more gene sequences encoding polypeptide A or B respectively.

10. A pair of plants as claimed in any one of claims 3 to 9 wherein the cause of male-sterility is direct or indirect.

11. A pair of plants as claimed in any one of claims 5 to 10 wherein the tissue-specific promoter is a tapetum-specific promoter, an embryo-specific promoter or a seed specific promoter.

12. A pair of plants as claimed in any one of claims 1 to 11 wherein one or both of the polypeptides or proteins is fused to a carrier protein.

13. A pair of plants as claimed in any one of claims 1 to 12 wherein each polypeptide or protein A, B is linked to a protein dimerisation domain of a dimeric or multimeric protein sequence that promotes association of between subunits A and B.

14. A pair of plants as claimed in any one of the preceding claims wherein the one or more gene sequences from at least one of the parent plants is a heterologous gene sequence.

15. A method for producing a plant having a desired phenotype by virtue of an active enzyme, a regulatory protein or a protein which affects the structural integrity of a cell, the method comprising crossing a first line with a second line wherein the first line contains one or more gene sequences encoding a polypeptide or protein but which line does not have the desired phenotype and wherein the second line contains one or more gene sequences encoding a polypeptide or protein B which is complementary to the polypeptide or protein A but which line does not have the desired phenotype.

16. A method as claimed in claim 15 wherein the one or more gene sequences from at least one of the lines is transgenic.

17. A method as claimed in claim 15 or claim 16 wherein desired phenotype is cell ablation especially male-sterility or embryoless seeds.

18. A method as claimed in any one of claims 15 to 17 wherein one of the lines is male-sterile.

19. A method as claimed in any one of claims 15 to 18 wherein the one or more gene sequences encoding polypeptides or protein A and/or B is operatively linked to a tissue-specific promoter.

20. A method as claimed in any one of claims 15 to 19 wherein the polypeptides or proteins A, B are naturally occurring subunits of an active enzyme, regulatory protein or protein which affects the structural integrity of a cell.

21. A method as claimed in claim 20 wherein the polypeptides or proteins A, B are two polypeptide subunits of an enzyme having RNase activity such as the enzyme Barnase, RNase A or the subunits of the protein Apelata3-pistillata.

22. A method as claimed in any one of claims 15 to 19 wherein the polypeptides or proteins A, B are artificially split polypeptides of an active enzyme, regulatory protein/ or protein which affects the structural integrity of a cell.

23. A method as claimed in any one of claims 15 to 22 wherein each line is homozygous with respect to the gene sequence encoding polypeptide or protein A, B, respectively.

24. A method as claimed in any one of claims 15 to 23 wherein the desired phenotypic trait is direct or indirect male-sterility.

25. A method as claimed in any one of the claims 15 to 24 wherein the tissue-specific promoter is a tapetum-

specific promoter, an embryo-specific promoter or a seed specific promoter.

26. A method as claimed in any one of claims 15 to 25 wherein one or both of the polypeptides or proteins A, B is fused to a carrier protein.

27. A method as claimed in any one of claims 15 to 26 wherein each polypeptide or protein A, B is linked to a different protein dimerisation domain of a dimeric or multimeric protein.

28. A method as claimed in any one of claims 15 to 27 wherein at least one of the lines contains, as the one or more gene sequences, heterologous gene sequences.

29. A seed or plant obtainable from a pair of plants as claimed in any one of claims 1 to 14 or by a method as claimed in any one of claims 15 to 28.

30. A seed or plant, having a phenotype of an active enzyme, regulatory protein or protein which affects the integrity of a cell, which is caused by the combined action of two or more transgenes, not present on the same copy of a chromosome.

31. A pair of plants for producing seeds, substantially as hereinbefore described, with reference to example 2 or example 3.

32. A method for producing a plant having a desired phenotype, substantially as hereinbefore described, with reference to example 2 or example 3.

Figure 1

A Barnase coding sequence

```
1                    met ala gln val ile asn thr phe asp gly val ala asp tyr leu gln thr tyr
2           TCTAGACC ATG GCA CAG GTT ATC AAC ACG TTT GAC GGG GTT GCG GAT TAT CTT CAG ACA TAT
3   3'gttcatgagatctgg tac 5' (B1 primer)

1                    his lys leu pro asp asn tyr ile thr lys ser glu ala gln ala leu gly trp
2                    CAT AAG CTA CCT GAT AAT TAC ATT ACA AAA TCA GAA GCA CAA GCC CTC GGC TGG
3                                                        (B4 primer)  3'  t gtt cgg gag ccg acc5'

1                    val ala ser lys gly asn leu ala asp val ala pro gly lys ser ile gly gly
2                    GTG GCA TCA AAA GGG AAC CTT GCA GAC GTC GCT CCG GGG AAA AGC ATC GGC GGA
3                      5'gca tca aaa ggg aac c 3' (B2 primer)

1                    asp ile phe ser asn arg glu gly lys leu pro gly lys ser gly arg thr trp
2                    GAC ATC TTC TCA AAC AGG GAA GGC AAA CTC CCG GGC AAA AGC GGA CGA ACA TGG
3

1                    arg glu ala asp ile asn tyr thr ser gly phe arg asn ser asp arg ile leu
2                    CGT GAA GCG GAT ATT AAC TAT ACA TCA GGC TTC AGA AAT TCA GAC CGG ATT CTT
3

1                    tyr ser ser asp trp leu ile tyr lys thr thr asp his tyr gln thr phe thr
2                    TAC TCA AGC GAC TGG CTG ATT TAC AAA ACA ACG GAC CAT TAT CAG ACC TTT ACA
3

1                    lys ile arg OCH
2                    AAA ATC AGA taa
3
```

B Intergenic sequence

```
CGAAAAAAACGGCTTCCTGCGGAGGCCGTTTTTTTCAGCTTTACATAAAGTGTGTAATAAATTTTTCTTCAAACTCTGATCGGTCAATTT
CACTTTCCGGATCCGGTCCAATCTGCAGCCGTCCGAGACAGGAGGACATCGTCCAGCTGAAACCGGGGCAGAATCCGGCCATTTCTGAAG
AGAAAAATGGTAAACTGATAGAATAAAATCATAAGAAAGGAGCCGCAC
```

C Barstar coding sequence

```
1                    Met lys lys ala val ile asn gly glu gln ile arg ser ile ser asp leu his
2                    ATG AAA AAA GCA GTC ATT AAC GGG GAA CAA ATC AGA AGT ATC AGC GAC CTC CAC
3

1                    gln thr leu lys lys glu leu ala leu pro glu tyr tyr gly glu asn leu asp
2                    CAG ACA TTG AAA AAG GAG CTT GCC CTT CCG GAA TAC TAC GGT GAA AAC CTG GAC
3

1                    ala leu trp asp cys leu thr gly trp val glu tyr pro leu val leu glu trp
2                    GCT TTA TGG GAT TGT CTG ACC GGA TGG GTG GAG TAC CCG CTC GTT TTG GAA TGG
3

1                    arg gln phe glu gln ser lys gln leu thr glu asn gly ala glu ser val leu
2                    AGG CAG TTT GAA CAA AGC AAG CAG CTG ACT GAA AAT GGC GCC GAG AGT GTG CTT
3

1                    gln val phe arg glu ala lys ala glu gly cys asp ile thr ile ile leu ser
2                    CAG GTT TTC CGT GAA GCG AAA GCG GAA GGC TGC GAC ATC ACC ATC ATA CTT TCT
3

1                    OCH
2                    TAA TACGATCAATGGGAGATGAACAATATAGATCCCCCGGGCTGCAGGAATTC
3                  5'taa tacgatcaatgggagatg 3' (B3 primer)
```

1: Translation of DNA sequences encoding Barnase (A) and Barstar (C), respectively
2: DNA sequence encoding either Barnase (A), Barstar (C) or the synthetic intergenic region (B) according to Paul et al. (1992)
3: Sequence of DNA primers that were used for IPCR to construct pepA* (B3/B4) and pepB* (B1/B2).

Figure 2: Schematic Illustration of pepA* and pepB*construction by Inverse PCR (IPCR)

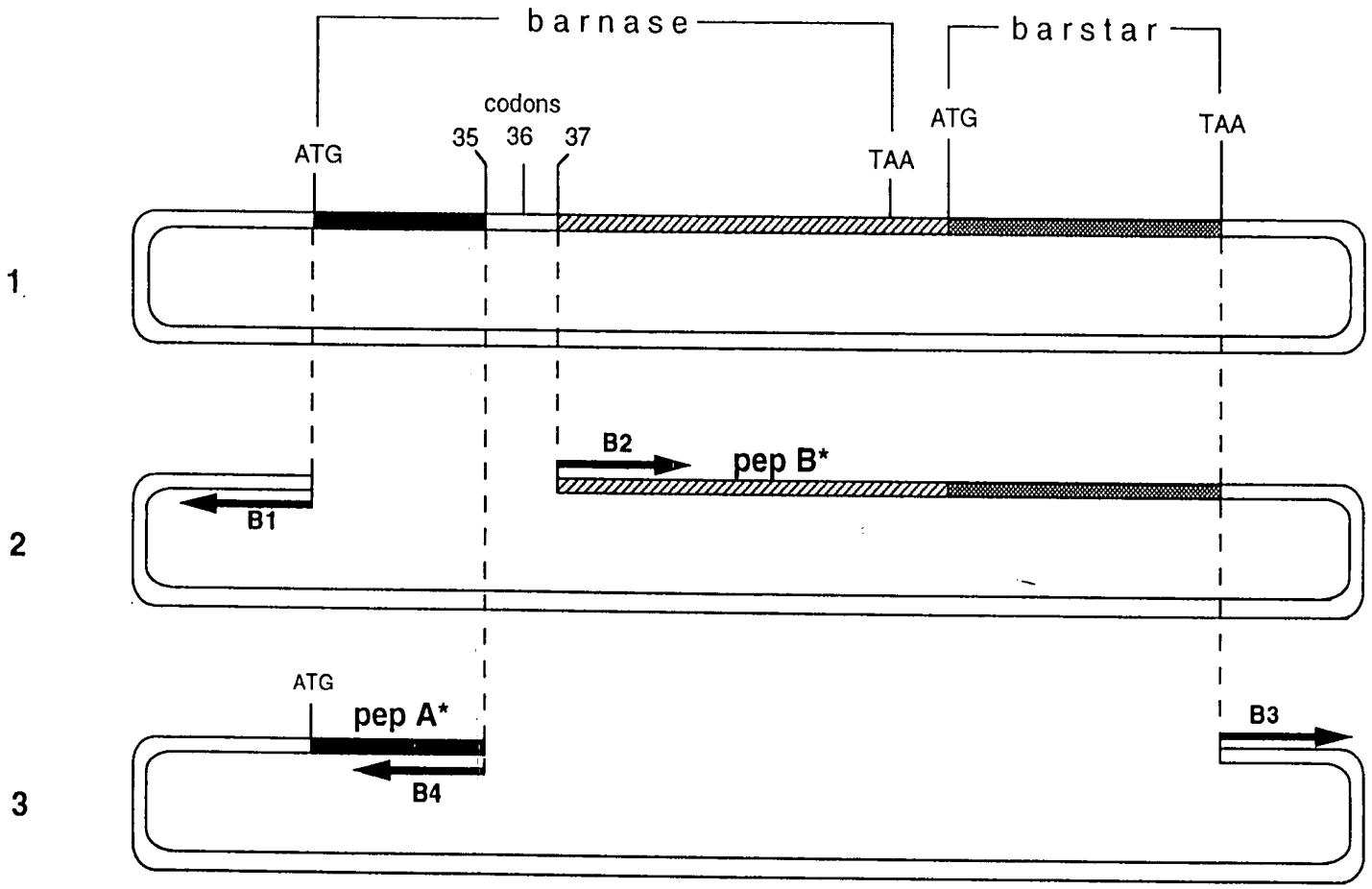


Figure 3 A: **In Vitro Construction from Synthetic Oligonucleotides of S-peptide, S(+5)-protein and S-protein**

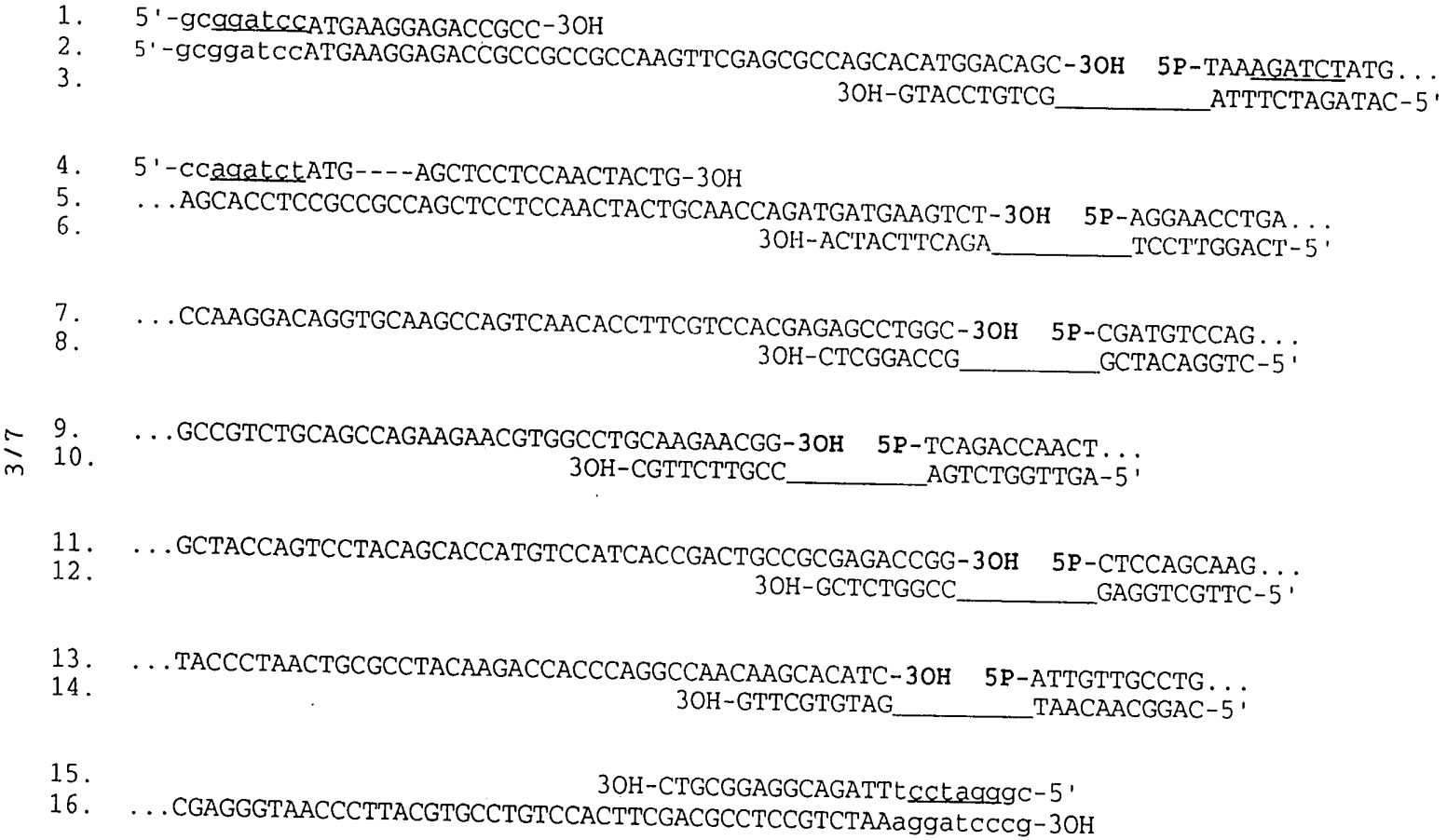


Figure 3 B: **In Vitro Construction from Synthetic Oligonucleotides of the Sequence encoding the S-peptide and the (Gly4-Ser)3 Linker**

1. 5'-gcggatccATGAAGGAGACCGCC-3OH
2. 5'-gcggatccATGAAGGAGACCGCCGCCGCCAAGTTCGAGCGCCAGCACATGGACAGC-3OH 5P-GGCGGTGG...
3. 3OH-GTACCTGTCG_____CCGCCACC-5'

4. ...CGGTTCCGGTGGCGGTGGCAGCGGCGGCGGTGGTAGCaagatcttcggg-3OH
5. 3OH-CCATCGttctagaagccc-5'

Figure 4 A

Protein and DNA Sequences of S-peptide and S-peptide with (Gly4 Ser)3 linker

```
1     ------ --- AAA GAG ACA GCA GCC GCA AAG TTT GAG CGT CAG CAT ATG GAT AGT --- --- --- --- --- ---
2     ------ MET lys glu thr ala ala ala lys phe glu arg gln his met asp ser OCH gly gly gly gly ser
3     ggatcc atg aag GAG acc gcc GCC gcc AAG ttc GAG cgc CAG cac ATG gac agc taa --- --- --- --- ---
4     ggatcc atg aag GAG acc gcc GCC gcc AAG ttc GAG cgc CAG cac ATG gac agc --- ggc ggt ggc ggt tcc

1           --- --- --- --- --- --- --- --- --- --- ------
2           gly gly gly gly ser gly gly gly gly ser ------
3           --- --- --- --- --- --- --- --- --- --- -agatct
4           ggt ggc ggt ggc agc ggc ggc ggt ggt agc aagatct
```

Legend to Figure 4 A:

1: DNA sequence of the synthetic Bovine Rnase A gene (codon 1 to 15) according to N. Vasantha and David Filpula (1989)
2: Translation of synthetic DNA sequences encoding Bovine RNase A
3: DNA sequence of the S-peptide coding sequence referred to in this invention
4: DNA sequence encoding the S-peptide with (gly4 ser)3 linker peptide referred to in this invention

Figure 4 B

Protein and DNA Sequences of S(+5)-protein and S-protein

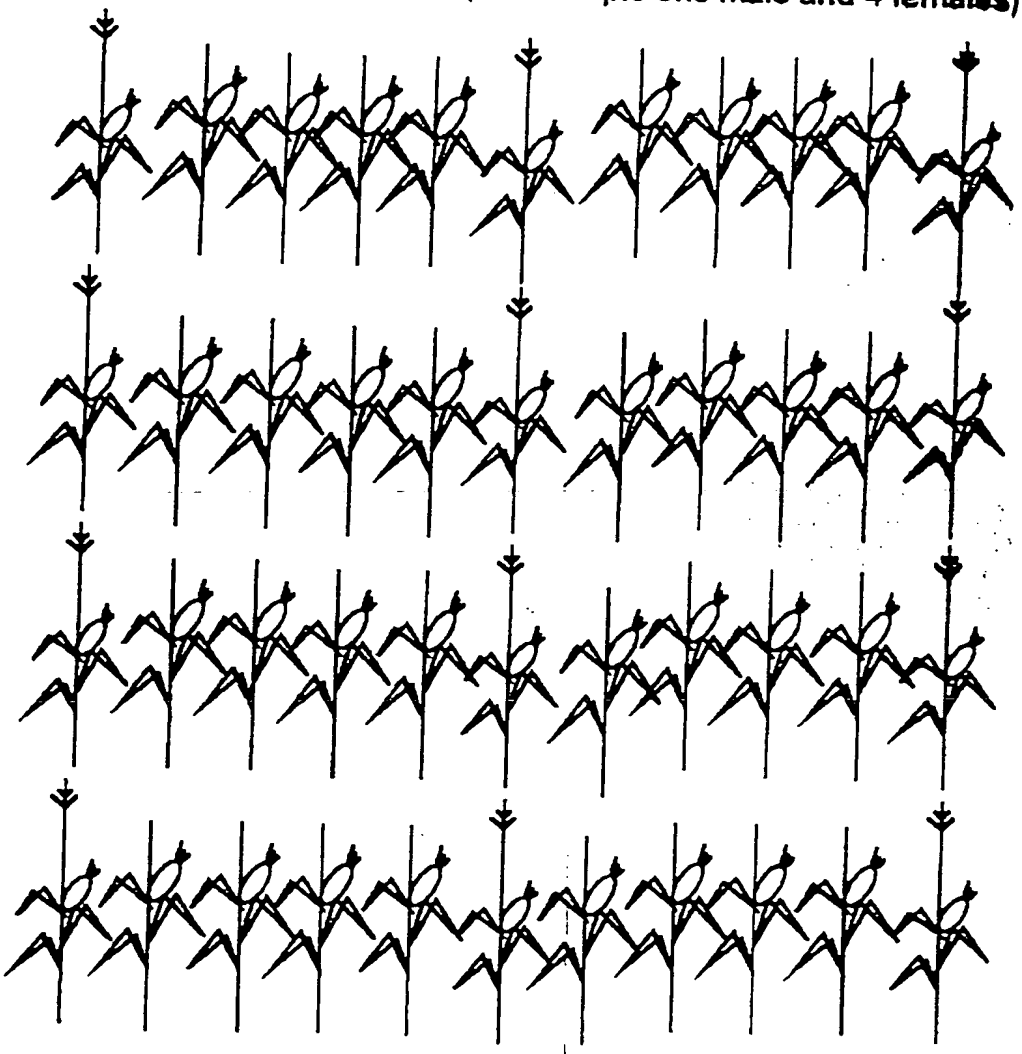
```
1           --- AGC ACC AGT GCT GCC AGT TCT TCC AAC TAC TGT AAC CAG ATG ATG AAG TCT AGA AAC TTG ACC AAG
2           met ser thr ser ala ala ser ser ser asn tyr cys asn gln met met lys ser arg asn leu thr lys
3     agatct atg AGC ACC tcc gcc GCC agc tcc TCC AAC TAC tgc AAC CAG ATG ATG AAG TCT agg AAC ctg ACC AAG
4     agatct atg --- --- --- --- --- agc tcc TCC AAC TAC tgc AAC CAG ATG ATG AAG TCT agg AAC ctg ACC AAG

1              GAC AGA TGT AAG CCA GTT AAC ACA TTT GTC CAC GAG AGT TTG GCT GAT GTC CAA GCC GTC TGC AGT
2              asp arg cys lys pro val asn thr phe val his glu ser leu ala asp val gln ala val cys ser
3              GAC agg tgc AAG CCA gtc AAC acc ttc GTC CAC GAG agc ctg gcc GAT GTC cag GCC GTC TGC agc
4              GAC agg tgc AAG CCA gtc AAC acc tcc GTC CAC GAG agc ctg gcc GAT GTC cag GCC GTC TGC agc

1              CAG AAA AAC GTT GCA TGC AAG AAC GGT CAA ACG AAC TGT TAC CAG AGT TAC AGC ACC ATG TCC ATC
2              gln lys asn val ala cys lys asn gly gln thr asn cys tyr gln ser tyr ser thr met ser ile
3              CAG aag AAC gtg gcc TGC AAG AAC GGT cag acc AAC tgc TAC CAG tcc TAC agc ACC ATG TCC ATC
4              CAG aag AAC gtg gcc TGC AAG AAC GGT cag acc AAC tgc TAC CAG tcc TAC agc ACC ATG TCC ATC

1              ACT GAC TGT CGT GAG ACA GGC TCG AGC AAG TAT CCT AAT TGT GCT TAC AAG ACC ACA CAG GCG AAC
2              thr asp cys arg glu thr gly ser ser lys tyr pro asn cys ala tyr lys thr thr gln ala asn
3              acc GAC tgc cgc GAG acc GGC tcc AGC AAG tac CCT aac tgc gcc TAC AAG ACC acc CAG gcc AAC
4              acc GAC tgc cgc GAG acc GGC tcc AGC AAG tac CCT aac tgc gcc TAC AAG ACC ACA CAG gcc AAC

1              AAA CAC ATC ATT GTT GCT TGT GAA GGT AAC CCT TAC GTT CCT GTC CAC TTT GAC GCC AGT GTT TAA
2              lys his ile ile val ala cys glu gly asn pro tyr val pro val his phe asp ala ser val OCH
3              aag CAC ATC ATT GTT gcc tgc gag GGT AAC CCT TAC gtg CCT GTC CAC ttc GAC GCC tcc gtc TAA
4              aag CAC ATC ATT GTT gcc tgc gag GGT AAC CCT TAC gtg CCT GTC CAC ttc GAC GCC tcc gtc TAA

1              -------
2              -------
3              aggatcc
4              aggatcc
```

Legend to Figure 4 B:

1: DNA sequence of the synthetic Rnase A gene (codons 16 to 124) according to Vasantha and Filpula (1989)
2: Translation of DNA sequences encoding the Bovine RNase A
3: DNA sequence of the synthetic S(+5)-protein coding sequence (aa16 to aa124)
4: DNA sequence of the synthetic S-protein coding sequence (aa21 to aa124)

Figure showing a production scheme of embryo less maize grains : Lines A and B are sown in alternativ rows (for xample one male and 4 females)

Figure 5



Embryoless seeds harvested from female rows only = 100% of embryoless maize seeds

OR

Seeds harvested from all the field plants = approximately 80% of embryoless maize seeds ;
note that if this sort of seeds harvesting is suited a random sowing with 10% of male plants and 90% of female plants is desirable and possible

Legend

male parent A
expressing pepA* in embryos
Genotype : emb-pepA* / emb-pepA*
or
emb-pepA* linked to Herbicide resistance/ emb-pepA* linked to herbicide resistance

female parent B
expressing pepB* in embryos only
Genotype : emb-pepB* / emb-pepB* in a male sterile cytoplasmic environment
or
emb-pepB* / emb-pepB*
Artificial Male Sterility linked to Herbicide Resistance / +

Figure 1

A Barnase coding sequence

```
1                    met ala gln val ile asn thr phe asp gly val ala asp tyr leu gln thr tyr
2              TCTAGACC ATG GCA CAG GTT ATC AAC ACG TTT GAC GGG GTT GCG GAT TAT CTT CAG ACA TAT
3     3'gttcatgagatctgg tac 5' (B1 primer)

1                    his lys leu pro asp asn tyr ile thr lys ser glu ala gln ala leu gly trp
2                    CAT AAG CTA CCT GAT AAT TAC ATT ACA AAA TCA GAA GCA CAA GCC CTC GGC TGG
3                                                          (B4 primer)  3'  t gtt cgg gag ccg acc5'

1                    val ala ser lys gly asn leu ala asp val ala pro gly lys ser ile gly gly
2                    GTG GCA TCA AAA GGG AAC CTT GCA GAC GTC GCT CCG GGG AAA AGC ATC GGC GGA
3                      5'gca tca aaa ggg aac c 3' (B2 primer)

1                    asp ile phe ser asn arg glu gly lys leu pro gly lys ser gly arg thr trp
2                    GAC ATC TTC TCA AAC AGG GAA GGC AAA CTC CCG GGC AAA AGC GGA CGA ACA TGG
3

1                    arg glu ala asp ile asn tyr thr ser gly phe arg asn ser asp arg ile leu
2                    CGT GAA GCG GAT ATT AAC TAT ACA TCA GGC TTC AGA AAT TCA GAC CGG ATT CTT
3

1                    tyr ser ser asp trp leu ile tyr lys thr thr asp his tyr gln thr phe thr
2                    TAC TCA AGC GAC TGG CTG ATT TAC AAA ACA ACG GAC CAT TAT CAG ACC TTT ACA
3

1                    lys ile arg OCH
2                    AAA ATC AGA taa
3
```

B Intergenic sequence

```
CGAAAAAAACGGCTTCCTGCGGAGGCCGTTTTTTTCAGCTTTACATAAAGTGTGTAATAAATTTTTCTTCAAACTCTGATCGGTCAATTT
CACTTTCCGGATCCGGTCCAATCTGCAGCCGTCCGAGACAGGAGGACATCGTCCAGCTGAAACCGGGGCAGAATCCGGCCATTTCTGAAG
AGAAAAATGGTAAACTGATAGAATAAAATCATAAGAAAGGAGCCGCAC
```

C Barstar coding sequence

```
1                    Met lys lys ala val ile asn gly glu gln ile arg ser ile ser asp leu his
2                    ATG AAA AAA GCA GTC ATT AAC GGG GAA CAA ATC AGA AGT ATC AGC GAC CTC CAC
3

1                    gln thr leu lys lys glu leu ala leu pro glu tyr tyr gly glu asn leu asp
2                    CAG ACA TTG AAA AAG GAG CTT GCC CTT CCG GAA TAC TAC GGT GAA AAC CTG GAC
3

1                    ala leu trp asp cys leu thr gly trp val glu tyr pro leu val leu glu trp
2                    GCT TTA TGG GAT TGT CTG ACC GGA TGG GTG GAG TAC CCG CTC GTT TTG GAA TGG
3

1                    arg gln phe glu gln ser lys gln leu thr glu asn gly ala glu ser val leu
2                    AGG CAG TTT GAA CAA AGC AAG CAG CTG ACT GAA AAT GGC GCC GAG AGT GTG CTT
3

1                    gln val phe arg glu ala lys ala glu gly cys asp ile thr ile ile leu ser
2                    CAG GTT TTC CGT GAA GCG AAA GCG GAA GGC TGC GAC ATC ACC ATC ATA CTT TCT
3

1                    OCH
2                    TAA TACGATCAATGGGAGATGAACAATATAGATCCCCCGGGCTGCAGGAATTC
3                  5'taa tacgatcaatgggagatg 3' (B3 primer)
```

1: Translation of DNA sequences encoding Barnase (A) and Barstar (C), respectively
2: DNA sequence encoding either Barnase (A), Barstar (C) or the synthetic intergenic region (B) according to Paul et al. (1992)
3: Sequence of DNA primers that were used for IPCR to construct pepA* (B3/B4) and pepB* (B1/B2).

Figure 2: Schematic Illustration of pepA* and pepB*construction by Inverse PCR (IPCR)

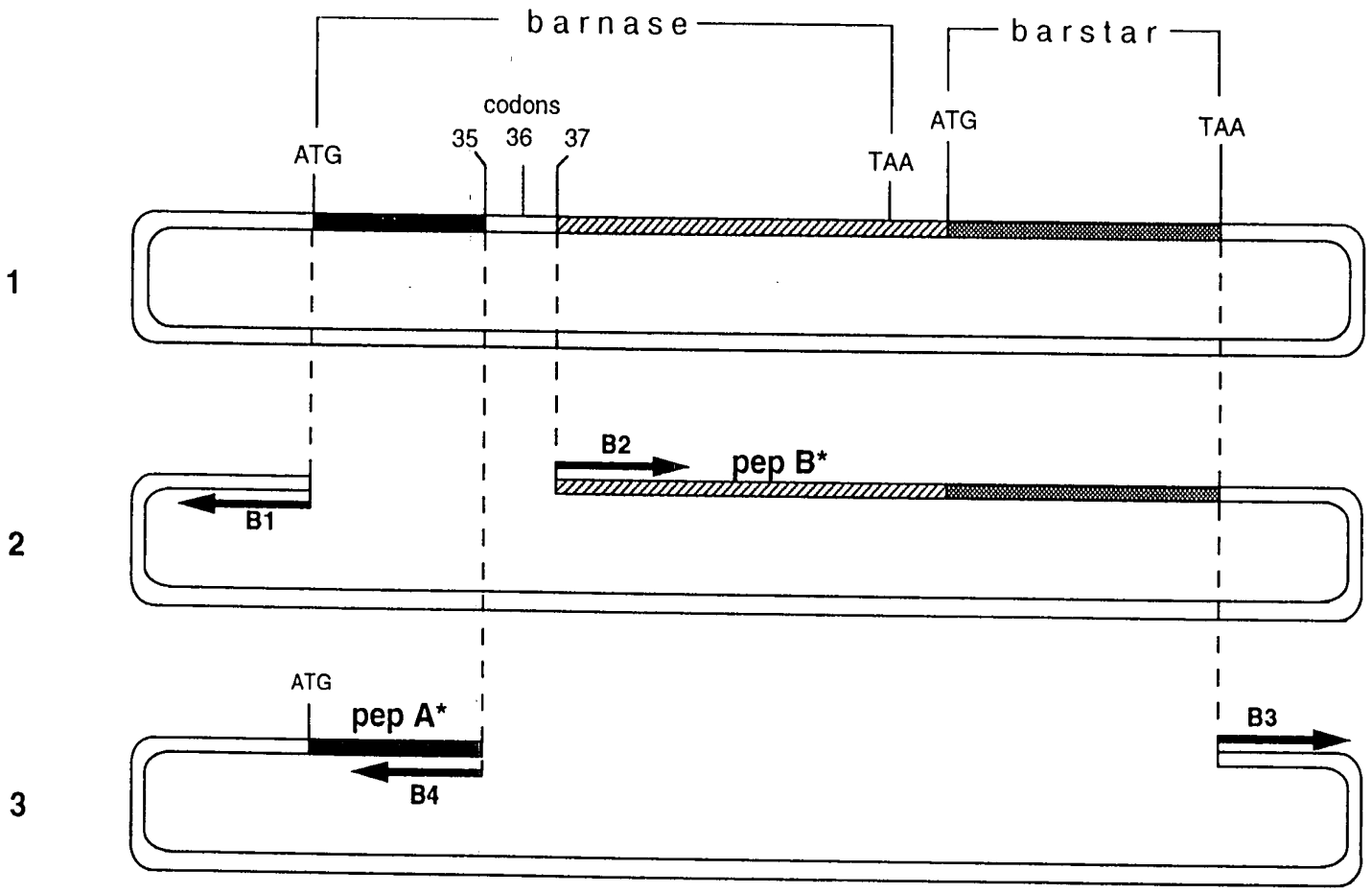


Figure 3 A: **In Vitro Construction from Synthetic Oligonucleotides of S-peptide, S(+5)-protein and S-protein**

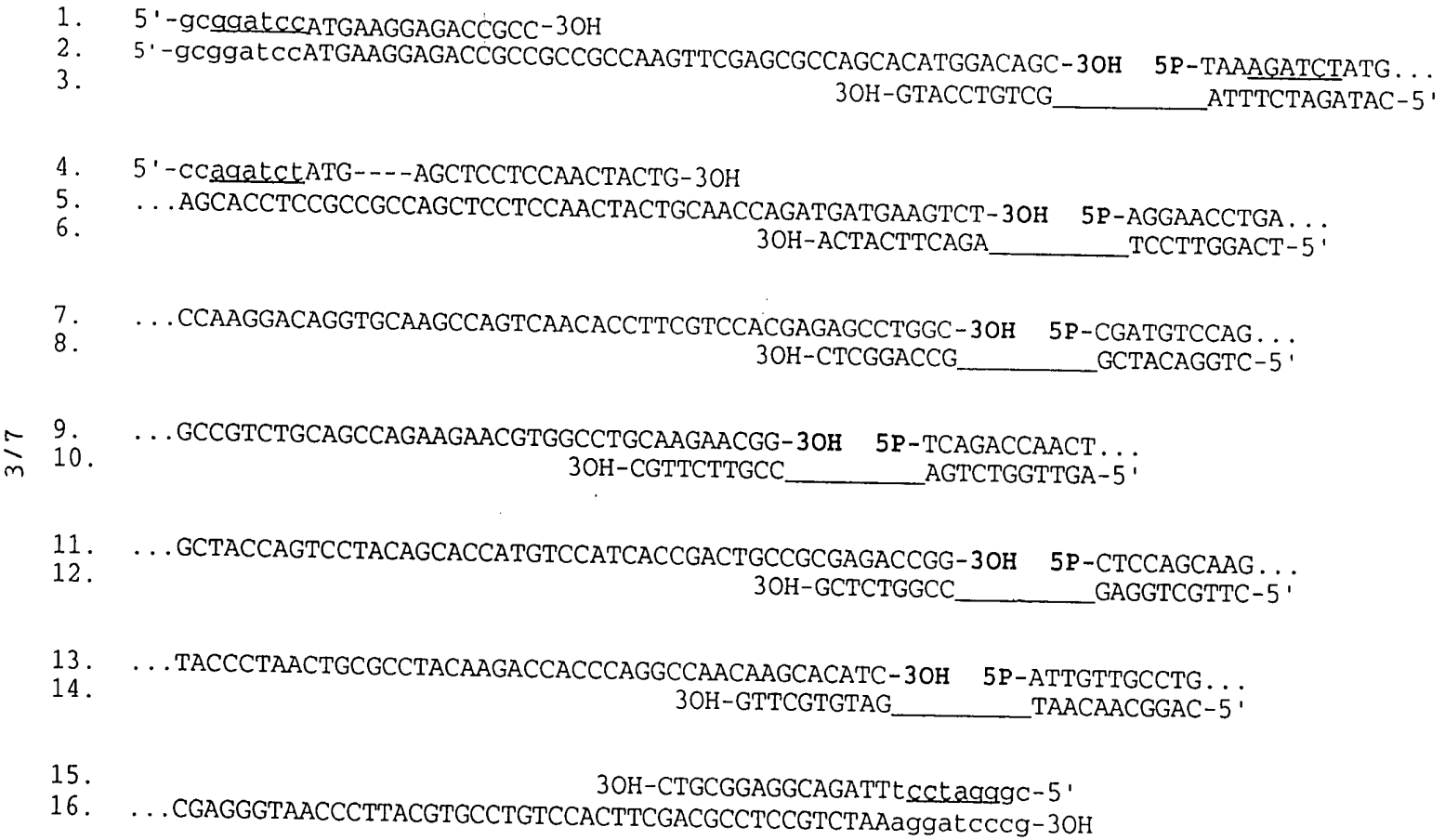


Figure 3 B: **In Vitro Construction from Synthetic Oligonucleotides of the Sequence encoding the S-peptide and the (Gly4-Ser)3 Linker**

```
1.   5'-gcggatccATGAAGGAGACCGCC-3OH
2.   5'-gcggatccATGAAGGAGACCGCCGCCGCCAAGTTCGAGCGCCAGCACATGGACAGC-3OH  5P-GGCGGTGG...
3.                                                          3OH-GTACCTGTCG_________CCGCCACC-5'

4.   ...CGGTTCCGGTGGCGGTGGCAGCGGCGGCGGTGGTAGCaagatcttcggg-3OH
5.                                  3OH-CCATCGttctagaagccc-5'
```

Figure 4 A

Protein and DNA Sequences of S-peptide and S-peptide with (Gly4 Ser)3 linker

```
1     ------ --- AAA GAG ACA GCA GCC GCA AAG TTT GAG CGT CAG CAT ATG GAT AGT --- --- --- --- --- ---
2     ------ MET lys glu thr ala ala ala lys phe glu arg gln his met asp ser OCH gly gly gly gly ser
3     ggatcc atg aag GAG acc gcc GCC gcc AAG ttc GAG cgc CAG cac ATG gac agc taa --- --- --- --- ---
4     ggatcc atg aag GAG acc gcc GCC gcc AAG ttc GAG cgc CAG cac ATG gac agc --- ggc ggt ggc ggt tcc

1           --- --- --- --- --- --- --- --- --- --- ------
2           gly gly gly gly ser gly gly gly gly ser ------
3           --- --- --- --- --- --- --- --- --- --- -agatct
4           ggt ggc ggt ggc agc ggc ggc ggt ggt agc aagatct
```

Legend to Figure 4 A:

1: DNA sequence of the synthetic Bovine Rnase A gene (codon 1 to 15) according to N. Vasantha and David Filpula (1989)
2: Translation of synthetic DNA sequences encoding Bovine RNase A
3: DNA sequence of the S-peptide coding sequence referred to in this invention
4: DNA sequence encoding the S-peptide with (gly4 ser)3 linker peptide referred to in this invention

Figure 4 B

Protein and DNA Sequences of S(+5)-protein and S-protein

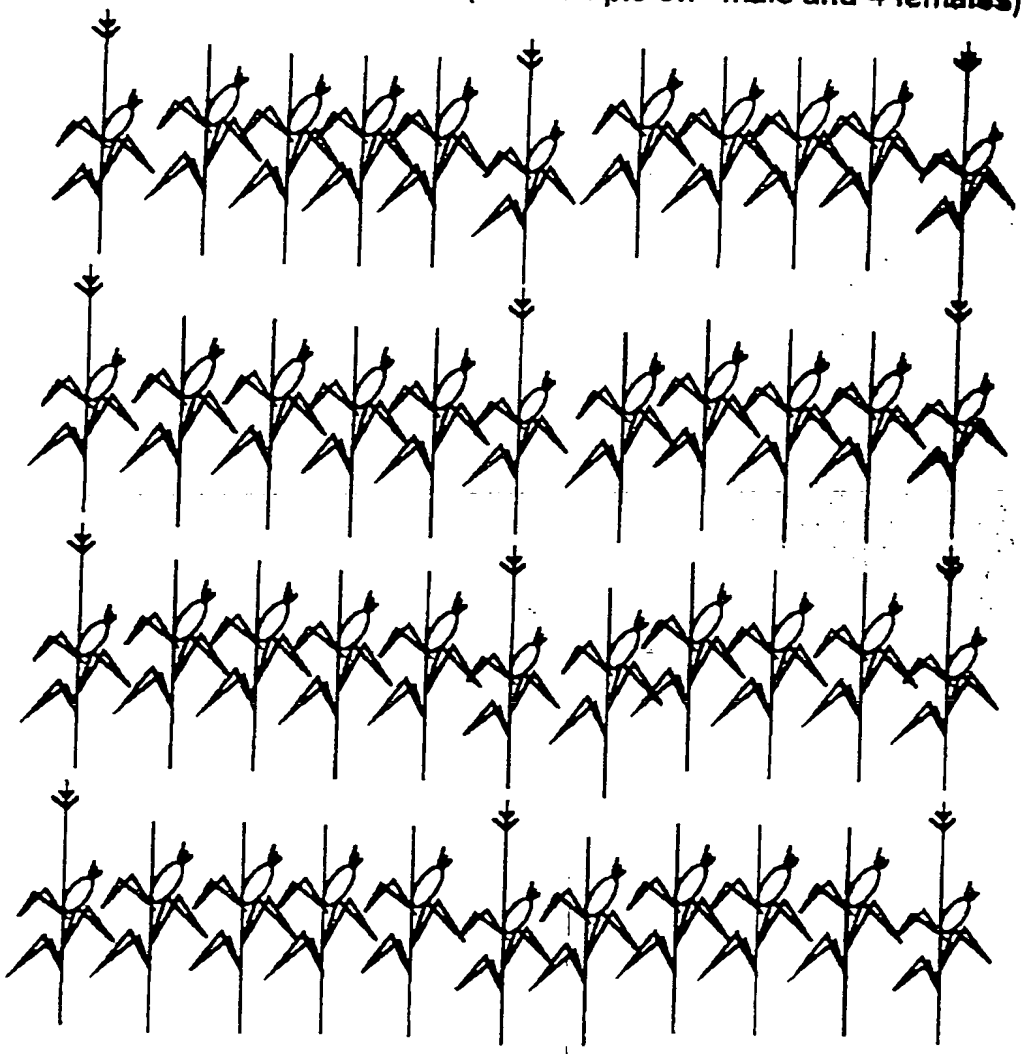
```
1           --- AGC ACC AGT GCT GCC AGT TCT TCC AAC TAC TGT AAC CAG ATG ATG AAG TCT AGA AAC TTG ACC AAG
2           met ser thr ser ala ala ser ser ser asn tyr cys asn gln met met lys ser arg asn leu thr lys
3    agatct atg AGC ACC tcc gcc GCC agc tcc TCC AAC TAC tgc AAC CAG ATG ATG AAG TCT agg AAC ctg ACC AAG
4    agatct atg --- --- --- --- --- agc tcc TCC AAC TAC tgc AAC CAG ATG ATG AAG TCT agg AAC ctg ACC AAG

1              GAC AGA TGT AAG CCA GTT AAC ACA TTT GTC CAC GAG AGT TTG GCT GAT GTC CAA GCC GTC TGC AGT
2              asp arg cys lys pro val asn thr phe val his glu ser leu ala asp val gln ala val cys ser
3              GAC agg tgc AAG CCA gtc AAC acc ttc GTC CAC GAG agc ctg gcc GAT GTC cag GCC GTC TGC agc
4              GAC agg tgc AAG CCA gtc AAC acc tcc GTC CAC GAG agc ctg gcc GAT GTC cag GCC GTC TGC agc

1              CAG AAA AAC GTT GCA TGC AAG AAC GGT CAA ACG AAC TGT TAC CAG AGT TAC AGC ACC ATG TCC ATC
2              gln lys asn val ala cys lys asn gly gln thr asn cys tyr gln ser tyr ser thr met ser ile
3              CAG aag AAC gtg gcc TGC AAG AAC GGT cag acc AAC tgc TAC CAG tcc TAC agc ACC ATG TCC ATC
4              CAG aag AAC gtg gcc TGC AAG AAC GGT cag acc AAC tgc TAC CAG tcc TAC agc ACC ATG TCC ATC

1              ACT GAC TGT CGT GAG ACA GGC TCG AGC AAG TAT CCT AAT TGT GCT TAC AAG ACC ACA CAG GCG AAC
2              thr asp cys arg glu thr gly ser ser lys tyr pro asn cys ala tyr lys thr thr gln ala asn
3              acc GAC tgc cgc GAG acc GGC tcc AGC AAG tac CCT aac tgc gcc TAC AAG ACC acc CAG gcc AAC
4              acc GAC tgc cgc GAG acc GGC tcc AGC AAG tac CCT aac tgc gcc TAC AAG ACC ACA CAG gcc AAC

1              AAA CAC ATC ATT GTT GCT TGT GAA GGT AAC CCT TAC GTT CCT GTC CAC TTT GAC GCC AGT GTT TAA
2              lys his ile ile val ala cys glu gly asn pro tyr val pro val his phe asp ala ser val OCH
3              aag CAC ATC ATT GTT gcc tgc gag GGT AAC CCT TAC gtg CCT GTC CAC ttc GAC GCC tcc gtc TAA
4              aag CAC ATC ATT GTT gcc tgc gag GGT AAC CCT TAC gtg CCT GTC CAC ttc GAC GCC tcc gtc TAA

1              -------
2              -------
3              aggatcc
4              aggatcc
```

Legend to Figure 4 B:

1: DNA sequence of the synthetic Rnase A gene (codons 16 to 124) according to Vasantha and Filpula (1989)
2: Translation of DNA sequences encoding the Bovine RNase A
3: DNA sequence of the synthetic S(+5)-protein coding sequence (aa16 to aa124)
4: DNA sequence of the synthetic S-protein coding sequence (aa21 to aa124)

Figure showing a production scheme of embryo less maize grains : Lines A and B are sown in alternative rows (for xample on male and 4 females)

Figure 5



Embryoless seeds harvested from female rows only = 100% of embryoless maize seeds

OR

Seeds harvested from all the field plants = approximately 80% of embryoless maize seeds ;
note that if this sort of seeds harvesting is suited a random sowing with 10% of male plants and 90% of female plants is desirable and possible

Legend

male parent A
expressing pepA* in embryos
Genotype : emb-pepA* / emb-pepA*
or
emb-pepA* linked to Herbicide resistance/ emb-pepA* linked to herbicide resistance

female parent B
expressing pepB* in embryos only
Genotype : emb-pepB* / emb-pepB* in a male sterile cytoplasmic environment
or
emb-pepB* / emb-pepB*
Artificial Male Sterility linked to Herbicide Resistance / +